

## Analysis of the Genetic Variability of Genes Encoding the RNA III-Activating Components Agr and TRAP in a Population of *Staphylococcus aureus* Strains Isolated from Cows with Mastitis

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The expression of *Staphylococcus aureus* virulence proteins is under the control of RNA III, a central pleiotropic regulator transcribed from the *agr* locus. RNA III is activated by at least two two-component systems, one encoded by the *agr* locus (AgrC-AgrA) and another encoded outside of this locus (TRAP-RAP). In this work, we developed new typing methods based on genes encoding these two systems, which we used to characterize a nonclonal population of *S. aureus* bovine mastitis isolates. Twelve *agr* restriction types were identified in this population, but the majority of strains (56.3%) were grouped in the R III-A1 type. No strain isolated from humans, whose *agr* sequence is available from GenBank, was found to belong to this major type. Restriction maps constructed for all of those *agr* variants allowed the linking of all types in an evolution scheme and their grouping in one of the four *agr* interference groups. This analysis indicates that groups 2, 3, and 4 probably evolved from the more frequently encountered type, which belongs to group 1. *agr* group 1 was also found to be the most prevalent (69.0% of the strains) and the most polymorphic interference group. By developing an *agr* group-specific multiplex PCR, we confirmed the above classification of strains in the *agr* interference groups. Four allelic variants of *trap* were also identified, indicating that this two-component system is also polymorphic. The majority of strains was grouped in the *trap* 1 type (71.8%). Whereas no relationships between *agr* group and *trap* types were found, strains of similar *agr* restriction type were also of similar *trap* type (with the exception of strains belonging to the *agr* R IV-A5 and R VI-A8 types). Our analysis indicates that *S. aureus* isolated from cows has predominantly a clonal structure and that the highly prevalent *agr* R III-A1, *trap* 1 type (56.3% of the strains) probably possesses a genetic background which endows it with superior ability to infect the bovine mammary gland.

*Staphylococcus aureus* is a gram-positive bacterium responsible for various major diseases in both humans and domestic animals. In dairy animals, *S. aureus* is one of the major causes of intramammary infections (mastitis) of lactating females, from whose milk it is frequently isolated. The presence of this bacterium in raw milk represents a risk for human health and causes serious economic losses to milk producers around the world (17, 27).

The pathogenesis of *S. aureus* is complex and involves both surface-associated proteins implicated in the adhesion of the bacterium to host tissues and the secretion of toxins that not only cause disease but also contribute to the bacterial spread (22). The expression of the virulence proteins is under the control of RNA III, a central pleiotropic regulator transcribed from the accessory gene regulator (*agr*) locus. RNA III is activated by at least two two-component systems, one (AgrC-AgrA) encoded by *agr* and another (target of RNA III-activating protein [TRAP]-RNA III-activating protein [RAP]) encoded outside of this locus (3, 22, 23).

The *agr* system is a quorum-sensing system that, during the

transition from the exponential to the stationary phase of growth, down-regulates the transcription of genes encoding some surface proteins and up-regulates the transcription of certain extracellular toxins (14, 22). The *agr* locus comprises two divergent transcriptional units, under the control of promoters P2 (RNA II) and P3 (RNA III). The P2 operon encodes a two-component signal transduction system (AgrC, transmembrane receptor-histidine kinase; AgrA, cytoplasmic regulator), a propeptide (AgrD), and an integral membrane protein (AgrB) that is probably involved in the processing and/or secretion of the peptide (Fig. 1). The resulting mature autoinducing peptide (AIP) accumulates in the extracellular environment during bacterial growth, reaches a threshold concentration (quorum sensing), and activates the two-component system by phosphorylation. The phosphorylated AgrA sensor then up-regulates the transcription from promoter P2, amplifying the response, and initiates transcription from promoter P3. The P3 transcript, RNA III, mediates up-regulation of secreted virulence factors as well as down-regulation of surface proteins (22). Interestingly, Ji et al. also described that AIP produced by a given strain of *S. aureus* activates its own *agr* locus but may inhibit the expression of *agr* in other strains. This phenomenon—due to polymorphism in a variable region of the *agr* locus comprising nucleotide sequences encoding AgrD, the C-terminal two-thirds of AgrB, and a portion of the N-terminal

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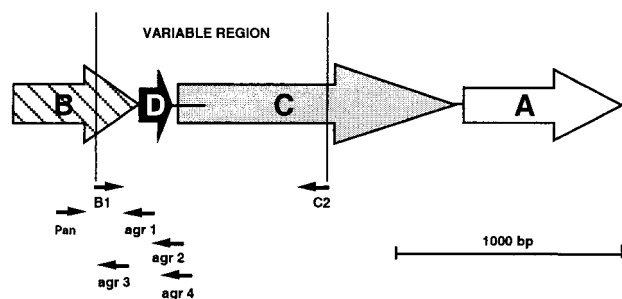


FIG. 1. Schematic map of the *S. aureus* *agr* operon (P2). The four members of the operon are represented with the position of the primers used in this work. *agrA* and *agrC* encode the response regulator and receptor-histidine protein kinase components of a two-component signal transduction pathway, whereas *agrB* and *agrD* combine to generate the activating ligand for the receptor. Large open arrows indicate the direction of transcription for each gene. Horizontal lines indicate intergenic regions (the usual length of the *agrD*-*agrC* intergenic region is 25 bp, but lengths up to 160 bp are also described). Thin arrows indicate the position of the primers used to amplify part of the operon. Primer lengths are not shown to scale, but their 5' ends are correctly positioned.

half of AgrC—actually led to the classification of *S. aureus* isolates in four different interference groups (12, 15). This type of bacterial interference could be implicated in the struggle for the colonization of infected sites (15).

On the other hand, RAP-TRAP, a second two-component system, was also shown to be able to activate transcription of RNA III (3). The proposed model taking into account this second system suggests that autoinduction of virulence occurs in a two-step process. At the beginning of the bacterial growth, the autoinducer RAP accumulates and induces the phosphorylation of the surface-associated protein TRAP. This results in up-regulation of the *agr* locus to produce RNA II. Once *agr* is activated (in the mid-exponential phase of growth), AIP and AgrC are produced. AIP accumulates in the environment and induces phosphorylation of AgrC, leading to phosphorylation of AgrA, up-regulation of RNA III synthesis, and down-regulation of TRAP phosphorylation (3, 32). Actually, it is not known if *trp* interstrain variation, similar to what was found for *agr*, does exist.

In this work, we developed new typing methods based on genes encoding the above-cited two-component systems, which we used to characterize the still poorly studied population of *S. aureus* strains isolated from cows with mastitis.

#### MATERIALS AND METHODS

**Bacterial strains.** A total of 71 *S. aureus* strains isolated from milk of cows with mastitis were analyzed. All strains were identified as *S. aureus* using standard microbiological techniques (10). These strains were isolated from different locations and at different times and were chosen to be epidemiologically unrelated. Most of the strains ( $n = 65$ ) were isolated from different regions of France by the Laboratory of Infectious Pathology and Immunology at the National Institute for Agronomic Research, but six strains were also isolated from different parts of the world: three strains were isolated in the United States (comprising strain 305 of Prasad and Newbould [ATCC 29470] [26]), one strain was isolated in the United Kingdom (the m strain of Neave and Oliver [ATCC 27543] [21]), and two strains were isolated in Japan (strains 125 and 130 of Takeuchi et al. [28]).

One strain was isolated in the 1950s, 15 strains were isolated in the 1960s, 16 strains were isolated in the 1970s, 12 strains were isolated in the 1980s, and 27 strains were isolated in the 1990s.

*S. aureus* *agr* reference strains RN6390 (*agr* group 1), RN6923 (*agr* group 2), RN8462 (*agr* group 3), and A880740 (*agr* group 4) (12, 15) and strains whose genomes are sequenced—N315 and Mu50 (18), COL (<http://www.tigr.org/tdb/mdb/mdbinprogress.html>), NCTC 8325 (<http://www.genome.ou.edu/staph.html>), and MRSA-252 and MSSA-476 ([http://www.sanger.ac.uk/Projects/S\\_aureus/](http://www.sanger.ac.uk/Projects/S_aureus/))—all from human origin, were used as controls.

**Nucleic acid purification.** For nucleic acid purification, strains were grown overnight on brain heart infusion agar plates at 37°C for 24 h. Ten to 15 colonies were then scraped from plates, and spheroplasts were prepared as described by van Leeuwen et al. (29). Nucleic acids were then purified from the spheroplasts as described by Boom et al. (4). Briefly, guanidine thiocyanate (catalog no. 50990; Fluka Biochemika) was added for cell lysis and the nucleic acids were purified by affinity chromatography with diatoms (high-purity, analytical grade Celite [catalog no. 16,743-6]; Aldrich). The nucleic acids were finally eluted from the diatom particles with 100  $\mu$ l of a  $10^{-4}$  M EDTA- $10^{-2}$  M Tris  $\cdot$  Cl (pH 8.0) solution and stored at  $-20^{\circ}\text{C}$  until use.

**PCR amplification of the variable region of the *agr* operon.** PCR amplification of the 1,070-bp variable region of the *agr* operon was performed with primers B1 (5'-TAT GCT CCT GCA GCA ACT AA-3') and C2 (5'-CTT GCG CAT TTC GTT GTT GA-3') described by van Leeuwen et al. (Fig. 1) (29). The variable *agr* region was amplified from 2  $\mu$ l of the purified nucleic acid solution in a 100- $\mu$ l reaction mixture containing 2.5 U of *Taq* DNA polymerase (*Taq* DNA polymerase in storage buffer A [Promega]), 200  $\mu$ M deoxynucleotide triphosphates (dNTPs) (Promega), 0.5  $\mu$ M primer B1, 0.5  $\mu$ M primer C2, 2 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1% Triton X-100, and 10 mM Tris  $\cdot$  Cl (pH 9.0). Amplifications were carried out in a Perkin-Elmer thermocycler (GeneAmp PCR system 9600) through the following temperature program: 1 cycle of 4 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 74°C; and finally 1 cycle at 74°C for 3 min. For some samples that were not amplified with the above protocol, the concentration of MgCl<sub>2</sub> in the PCR mixture was increased to 2.5 mM, and the annealing and elongation times were increased to 2 and 3 min, respectively. After precipitation with ethanol and centrifugation, the pellet was dissolved in 70  $\mu$ l of a  $10^{-4}$  M EDTA- $10^{-2}$  M Tris  $\cdot$  Cl (pH 9.0) solution. All samples were stored at  $-20^{\circ}\text{C}$  before restriction.

***agr* group-specific multiplex PCR.** The *agr* sequences were amplified from 2  $\mu$ l of the purified nucleic acid solutions in a 25- $\mu$ l reaction mixture containing 1.25 U of *Taq* DNA polymerase (*Taq* DNA polymerase in storage buffer A [Promega]), 200  $\mu$ M dNTPs (Promega), 5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1% Triton X-100, 10 mM Tris  $\cdot$  Cl (pH 9.0), and a 0.3  $\mu$ M concentration of each of the following primers: Pan (5'-ATG CAC ATG GTG CAC ATG C-3'), agr1 (5'-GTC ACA AGT ACT ATA AGC TGC GAT-3'), agr2 (5'-TAT TAC TAA TTG AAA AGT GGC CAT AGC-3'), agr3 (5'-GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G-3'), and agr4 (5'-CGA TAA TGC CGT AAT ACC CG-3'). These primers allow the amplification of a 441-bp DNA fragment of the *agr* group 1 strains, of a 575-bp DNA fragment of the *agr* group 2 strains, of a 323-bp DNA fragment of the *agr* group 3 strains, and of a 659-bp DNA fragment of the *agr* group 4 strains. Amplifications were carried out in an MJ Research thermocycler (PTC-100) through the following temperature program: 1 cycle of 5 min at 94°C; 26 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C; and finally 1 cycle of 72°C for 10 min. Amplification products were electrophoresed in a 1.5% agarose gel containing ethidium bromide and visualized by transillumination under UV.

**PCR amplification of the *trp* gene.** The entire 504-bp open reading frame of the *trp* gene was amplified with sense primer 5'-ACA TAA GGG GGA CCT TTC AT-3' (ending 1 nucleotide before the start codon) and antisense primer 5'-ACC AAT GGA AGT TTT CTT CG-3' (ending 4 nucleotides after the stop codon). The *trp* open reading frame was amplified from 2  $\mu$ l of the purified nucleic acid solutions in a 100- $\mu$ l reaction mixture containing 1.25 U of *Taq* DNA polymerase (*Taq* DNA polymerase in storage buffer A [Promega]), 200  $\mu$ M dNTPs (Promega), 1  $\mu$ M sense primer, 1  $\mu$ M antisense primer, 1 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1% Triton X-100, and 10 mM Tris  $\cdot$  Cl (pH 9.0). Amplifications were carried out in a Perkin-Elmer thermocycler (GeneAmp PCR system 9600) through the following temperature program: 1 cycle of 45 s at 95°C; 35 cycles of 45 s at 95°C, 60 s at 52°C, and 60 s at 72°C; and finally 1 cycle at 72°C for 10 min. All samples were stored at  $-20^{\circ}\text{C}$  before restriction.

**Restrictions of the PCR products.** The *trp* amplicons were restricted with *Mse*I (New England Biolabs), whereas the *agr* amplicons were restricted with *Rsa*I (Roche Molecular Biochemicals) and *Alu*I (Roche Molecular Biochemicals), according to manufacturer's instructions. The restriction fragments were then separated by electrophoresis on a 3% agarose gel (SeaKem HGT agarose; FMC, Rockland, Maine) containing ethidium bromide and visualized by transillumination under UV.

TABLE 1. Characteristics of different restriction types of the *agr* operon identified among the analyzed *S. aureus* strains isolated from cows with mastitis

<i>agr</i> type	<i>agr</i> group <sup>a</sup>	<i>trap</i> type(s)	No. of occurrences ( <i>n</i> = 71)	Lengths <sup>b</sup> (bp) of <i>agr</i> restriction fragments	
				<i>Rsa</i> I	<i>Alu</i> I
R I-A6	4	2	1	642, 275, 155	626, 184, 155, 107
R I'-A1	1	1	3	616, 276, 149, 32	274, 179, 168, 159, 155, 113, 25
R II-A1	1	1	1	320, 276, 174, 149, 122, 32	274, 179, 168, 159, 155, 113, 25
R III-A1	1	1	40	442, 276, 174, 149, 32	274, 179, 168, 159, 155, 113, 25
R III-A2	1	2	1	442, 276, 174, 149, 32	274, 268, 179, 168, 159, 25
R IV-A5	2	1, 3	9	484, 308, 280	502, 244, 149, 149, 28
R IV-A7	2	2	6	484, 308, 280	679, 244, 149
R V-A4	1	2	3	442, 323, 276, 32	442, 179, 159, 155, 113, 25
R VI-A8	3	2, 3	2	478, 287, 230, 79	772, 155, 74, 73
R VII-A3	1	1	1	442, 250, 50	325, 179, 159, 50, 25
R VIII-A12	1→3	3	1	442, 230, 174, 149, 79	274, 179, 159, 155, 135, 74, 73, 25
R IX-A11	1→2	2	1	442, 308, 174, 149	274, 244, 192, 179, 159, 25
Not amplifiable	2	4	2	Not applicable	Not applicable

<sup>a</sup> Strains were classified in one of the four *agr* groups by *agr* group-specific PCR and *agr* restriction map analysis. The putative evolving stage between two groups is indicated by an arrow.

<sup>b</sup> Lengths of restriction fragments were estimated by electrophoresis in the presence of molecular weight markers but, for some types, were also (R I-A6, GenBank accession no. AF288215; R I'-A1, GenBank accession no. X52543 [strain COL at The Institute for Genomic Research data bank and strain NCTC 8325 at the University of Oklahoma's ACGT data bank]; R III-A2, GenBank accession no. AF210055; R VI-A8, strain MSSA-476 at the Sanger data bank) or only (R VIII-A12, GenBank accession no. AB043555; R IX-A11, GenBank accession no. AB043554) calculated from computer-generated restrictions of *agr* nucleic acid sequences deposited in data banks.

## RESULTS

***agr* restriction fragment length polymorphism.** The polymorphism of the *agr* operon was analyzed by restriction endonuclease PCR in a population of 71 epidemiologically unrelated *S. aureus* bovine mastitis isolates. To this end, the 1,070-bp variable region of the *agr* operon was amplified by PCR with primers B1 and C2 (Fig. 1). Sixty-eight of the tested strains gave an amplicon of the expected molecular weight, and one strain gave an amplicon of around 740 bp, whereas no amplicon could be amplified from two strains (results not shown). The PCR products were then restricted with *Rsa*I or *Alu*I, giving 10 different profiles for each enzyme (Table 1; Fig. 2). The combination of these two restriction patterns allows the definition of 12 unique *agr* restriction types (Table 1). Most of the strains belong to the R III-A1 type (56.3%), the R IV-A5 type (12.7%), and the R IV-A7 type (8.4%). The other nine restriction types are shared by only one to three strains (Table 1). The numerical index of the discriminatory ability of the *agr* typing, calculated as described by Hunter and Gaston (11), indicates that if two strains were sampled randomly from the analyzed populations, then on 66.2% of occasions they would fall into different restriction types.

**Relationship between restriction types of the *agr* operon.** Restriction maps were constructed to know the relationship between the unique restriction types of the *agr* locus identified in the collection of strains isolated from cows with mastitis. To this end, partial hydrolysis was also conducted to correctly assign the position of some restriction fragments (results not shown). The maps were then compared with those corresponding to the same region of the *agr* operon sequenced from strains isolated from humans and available from nucleic acid databases (Fig. 3). The *agr* interference group reference strains isolated from humans were found to be type R I'-A1 for group 1 (GenBank accession no. X52543), type R IV-A9 for group 2 (GenBank accession no. AF001782), type R VI-A10 for group 3 (GenBank accession no. AF001783), and type R I-A6 for

group 4 (GenBank accession no. AF288215). Strain CMRSA-1 (GenBank accession no. AF210055) classified by Papakyriacou et al. (25) as being a variant of the *agr* interference group 1 reference strain (Ia) was identified to be type R III-A2. It is worth noting that no type R VI-A10 and R IV-A9 were identified among the strains isolated from cows with mastitis. Similarly, after amplification and restriction of the *agr* operon, the restriction maps of six strains of human origin whose genomes are sequenced were also constructed. Strains N315 and Mu50 are type R IV-A9, strains NCTC 8325 and COL are type R I'-A1, strain MRSA-252 is type R VI-A10, and strain MSSA-476 is type R VI-A8.

All restriction types were classified in one of the four *agr* groups on the basis of the presence or absence of a combination of restriction sites characteristic of each of the interference group reference sequences (Fig. 3 [types in grey]). All types were then linked one to the other inside each group, trying to produce the least gain or loss of restriction sites when evolving from one type to another (Fig. 3). This analysis allows the classification of all *agr* restriction types into the four previously known *agr* groups (Table 1). Nevertheless, it is of interest that the 3' extremity of the variable region of the *agr* locus (plus or minus two-thirds of the molecules) of two strains isolated from cows in Japan, the strain 125 (type R IX-A11) and the strain 130 (type R VIII-A12), possess the characteristic restriction sites of the *agr* group 1 strains, whereas the 5' extremity of this region (plus or minus one-third of the molecule) contains restriction sites characteristic of the *agr* group 2 and of the *agr* group 3 strains, respectively (Fig. 3). As the *agr* loci of both strains were sequenced (GenBank accession no. AB043555 and AB043554), this property was verified by aligning the nucleotide sequences of their variable *agr* region with those of the *agr* group 1 to 3 reference strains (results not shown).

The entire *agr* variable region of strains 125 and 130 being nevertheless more similar to the *agr* group 1 reference se-



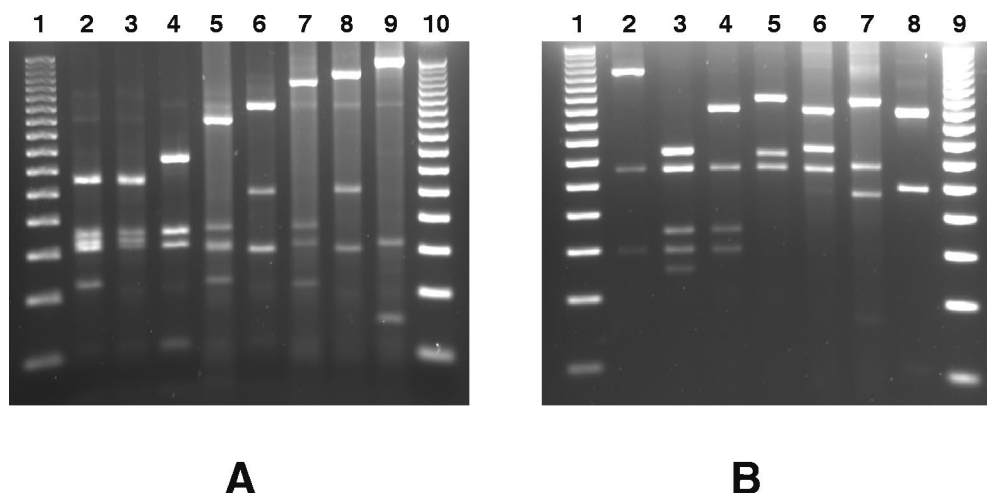


FIG. 2. Restriction polymorphism in the *agr* variable region of *S. aureus* strains isolated from cows with mastitis. The amplified variable region of the *agr* operon was digested with *AluI* (A) or *RsaI* (B) and electrophoresed on a 3% agarose gel. Examples of the different restriction types obtained are shown: type A1 (lane A2), type A2 (lane A3), type A3 (lane A4), type A4 (lane A5), type A5 (lane A6), type A6 (lane A7), type A7 (lane A8), type A8 (lane A9), type R I (lane B2), type R II (lane B3), type R III (lane B4), type R IV (lane B5), type R V (lane B6), type R VI (lane B7), and type R VII (lane B8). Type R I', being difficult to differentiate from type R I in a 3% agarose gel, is not shown. Types R VIII, R IX, A11, and A12 were identified by computer analysis of sequences with GenBank accession no. AB043554 and AB043555. Molecular weight markers (50-bp DNA ladder; Promega) are shown in lanes A1, A10, B1, and B9.

quence (88.2 and 91.1% of nucleotides identical, respectively) than to the *agr* reference sequences of group 2 (69.7 and 59.0% of nucleotides identical, respectively) or group 3 (64.9 and 76.0% of nucleotides identical, respectively), we tentatively classified these two strains in *agr* group 1, at the junction between groups 2 and 3, respectively (Fig. 3). Our analysis also showed that the isolate giving an *agr* amplicon of reduced length is an *agr* group 1 strain (type R VII-A3 [Fig. 3]) with a deletion of a 330-bp region comprising the entire *agrD* gene.

The restriction map analysis indicates that group 1 is the most diverse *agr* group. This group contains 8 out of the 14 restriction types identified, whereas group 2, group 3, and group 4 contain only 3 types, 2 types, and 1 type, respectively. Finally, we found that the sequenced human strains COL and NCTC 8325 belong to *agr* group 1, that strains N315 and Mu50 belong to *agr* group 2, and that strains MRSA-252 and MSSA-476 belong to *agr* group 3 (Fig. 3).

**Development of an *agr* group-specific multiplex PCR.** The analysis of the restriction maps of the variable region of the *agr* operon (Fig. 3) suggests that *agr* group-specific primers could be found in this region and used in an *agr* group-specific multiplex PCR. We thus analyzed in detail the nucleotide sequences of each of the four different *agr* operons obtained from GenBank (accession no. X52543, AF001782, AF001783, and AF288215) by using the Gene Jockey software (Biosoft, Cambridge, United Kingdom). One consensus forward primer (Pan) and four group-specific reverse primers (*agr1*, *agr2*, *agr3*, and *agr4*), which would allow the identification of the *agr* group on the basis of the molecular weight of its PCR product, were identified (Fig. 1). These primers were then tested experimentally in a multiplex PCR and shown to correctly identify the *agr* group of the *agr* reference strains RN6390 (group 1), RN6923 (group 2), RN8462 (group 3), and A880740 (group 4) (Fig. 4). We then used the above multiplex PCR to analyze the population of strains isolated from cows with mastitis. All of the 71

strains but 1 gave an amplifiable product. The latter strain is the one giving an *agr* amplicon of reduced length and was shown by restriction analysis to have a deletion of the sequence corresponding to the *agr* group 1- and group 3-specific primers (Fig. 3). As the 3' extremity of this amplicon possesses the characteristic restriction sites of group 1 strains and not those of group 3 strains, we definitely classify this strain in the *agr* group 1.

The *agr* group-specific multiplex PCR confirms the above *agr* group classification of strains made by restriction analysis. Nevertheless, strains 125 and 130 isolated in Japan, tentatively classified by restriction analysis in group 1, are now classified by multiplex PCR in groups 2 and 3, respectively. This is due to the fact that primers *agr2* and *agr3* are not localized in the 3' extremity of the *agr* variable region, which for these two strains is characteristic of group 1. These two strains are probably in a process of evolution from group 1 to groups 2 and 3, respectively. The classification of all six human strains used as controls into *agr* groups was also confirmed. The majority of strains isolated from cows with mastitis belong to *agr* group 1 (69.0%) and to *agr* group 2 (23.9%). Groups 3 and 4 contain only 2.8 and 1.4% of the analyzed strains, respectively. As discussed above, the classification of two strains (strains 125 and 130) is uncertain.

***trap* restriction fragment length polymorphism.** As TRAP has been proved to be a component of a membrane-associated sensor able to induce the synthesis of RNA III via a signal transduction pathway other than *agr*, we analyzed whether *trap* interstrain variation, similar to that which was found for *agr*, does exist. To this end, we first aligned the *trap* sequences of six strains whose genomes are sequenced. Theoretical restrictions with *MseI* (a frequently cutting enzyme in *trap*) show that the *agr* group 1 or 2 strains COL, NCTC 8325, N315, and Mu50 have a similar *MseI* restriction profile and that this profile is different from those of the *agr* group 3 strains MRSA-252 and

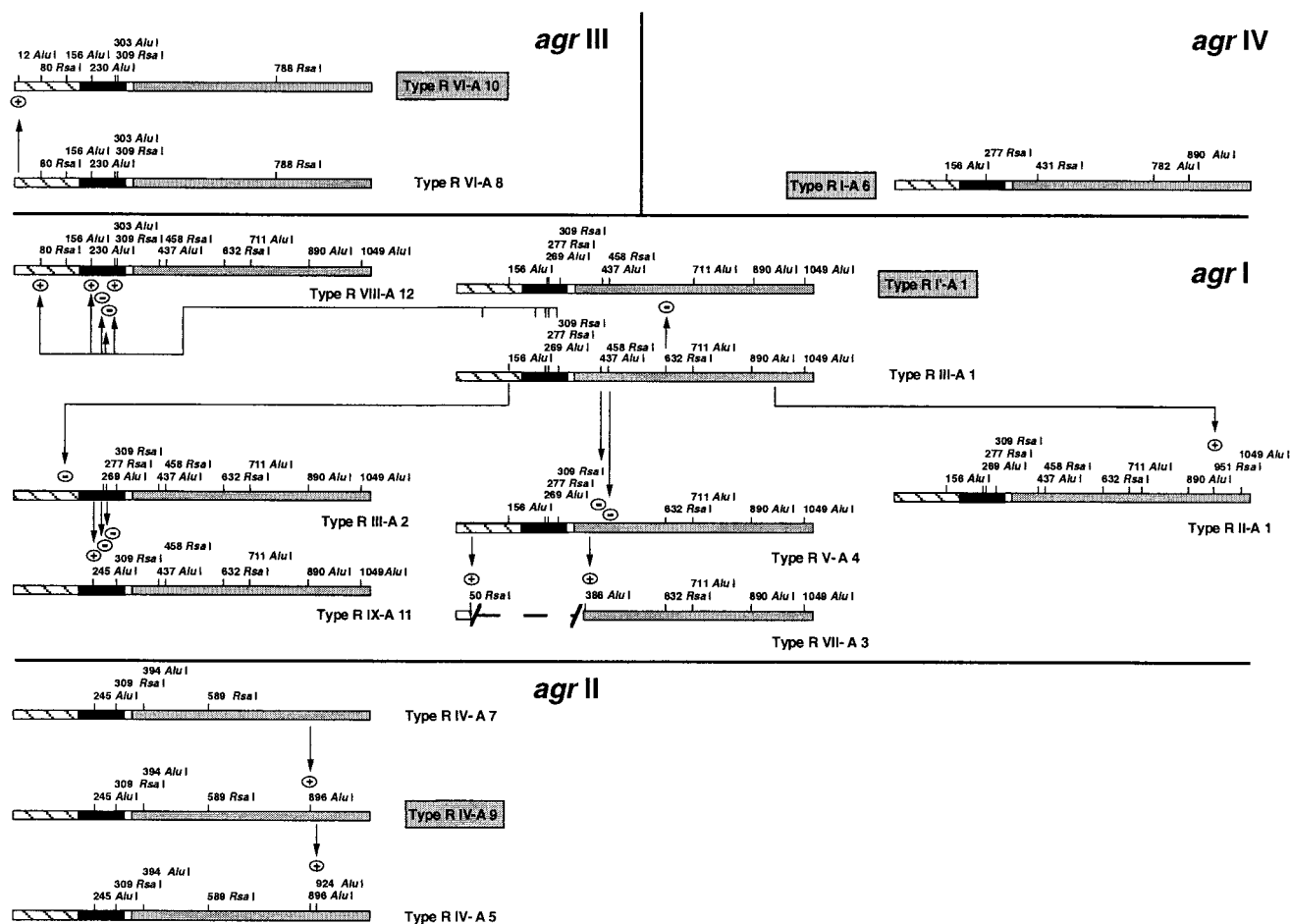


FIG. 3. Relationship between distinct restriction types of the *agr* operon identified among *S. aureus* strains isolated from cows with mastitis. The amplified variable region of the *agr* operon was digested with *AluI* and *RsaI*, and maps corresponding to the distinct restriction types identified in the population were constructed (3' end of *agrB* [striped bars], *agrD* [solid bars], intergenic region [open bars], 5' end of *agrC* [shaded bars]). These maps were compared with those corresponding to the same region of the *agr* group 1 to 4 reference sequences (type names in grey) obtained from GenBank (accession no. X52543 for group 1, accession no. AF001782 for group 2, accession no. AF001783 for group 3, and accession no. AF288215 for group 4). As the sequences of the *agr* operon deposited in GenBank do not always show any highly obvious translational start, the drafted length of the intergenic region represents the most frequently described length (25 bp). Based on the presence or absence of characteristic restriction sites, all restriction types were classified in one of the four *agr* groups. The arrows indicate the appearance (+) or disappearance (-) of a restriction site with respect to the other types of the same *agr* group. The dashed line indicates the position of a deletion in the operon.

MSSA-476 (results not shown). These preliminary results indicate that *trap* is polymorphic and suggest that a relation between *trap* types and *agr* group could perhaps exist. This prompted us to analyze the entire *S. aureus* population isolated from cows with mastitis for *trap* polymorphism.

*trap* genes were amplified from all strains by PCR and restricted with *MseI*. This allowed the identification of four different restriction types among the analyzed population (Fig. 5). The vast majority of strains isolated from cows belong to *trap* type 1 (71.8%). Types 2, 3, and 4 account for 18.3, 7.0, and 2.8% of the analyzed strains, respectively (Table 1). It is worth noting that we were previously unable to amplify the variable region of the *agr* locus in the only two strains of *trap* type 4 identified (Table 1). The human sequenced strains COL, NCTC 8325, Mu50, and N315 belong to *trap* type 1, whereas strains MRSA-252 and MSSA-476 belong to *trap* types 2 and 3, respectively.

No particular relationship between unique *agr* group and *trap* type could be displayed. Indeed, our experiments showed that the *agr* groups 2, 1, and 3 contain strains of *trap* types 1 to 4, strains of *trap* types 1 to 3, and strains of *trap* types 2 and 3, respectively. The *agr* group 4 contains a unique strain of *trap* type 2 (Table 1). Whereas no relationship was found between *agr* groups and *trap* types, strains possessing a similar *agr* restriction type were also found to possess a similar *trap* type. *Agr* types R IV-A5 (containing strains of *trap* types 1 and 3) and R VI-A8 (containing strains of *trap* types 2 and 3) are nevertheless an exception to this rule (Table 1).

## DISCUSSION

Polymorphism in the *agr* locus was first described by Ji et al. (15). This led to the classification of *S. aureus* isolates into four different interference groups (12, 15). Later, sequence varia-

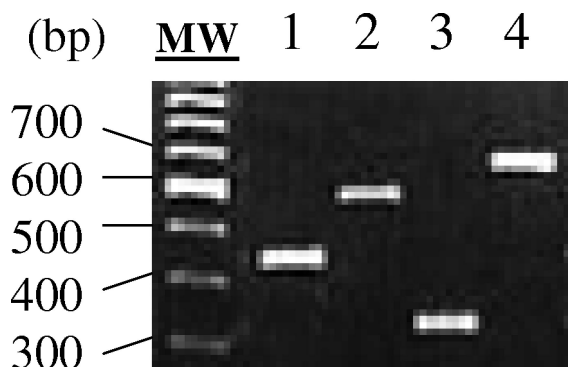


FIG. 4. *agr* group-specific multiplex PCR. *S. aureus* DNA from each of the four *agr* reference strains (RN6390 [group 1, lane 1], RN6923 [group 2, lane 2], RN8462 [group 3, lane 3], A880740 [group 4, lane 4]) were amplified with multiplex primers Pan, *agr*1, *agr*2, *agr*3, and *agr*4. PCR products were separated on a 1.5% agarose gel and visualized under UV.

tions within groups were also found (20, 25, 28, 29). In this work, we identified 14 different *agr* restriction types among the analyzed strains. Nevertheless, only 12 of them were present in our collection of bovine mastitis isolates. On the basis of the restriction maps of the *agr* variable region, we classified all *agr* types in one of the four interference groups (Fig. 3). This classification was confirmed by the *agr* group-specific PCR also developed in this work. Whereas strains belonging to each of the four *agr* groups were found, most of them (69.0%) were assigned to group 1. This repartition of strains among interference groups is quite similar to what was described by Moore and Lindsay for methicillin-sensitive hospital strains (19). The restriction sites used as markers to discriminate *agr* alleles indicate that the *agrD* sequences are stable within each interference group. Most mutations within groups appear to arise in

*agrC*, the gene encoding the receptor of the AIP. Those mutations are probably not in sequences coding for amino acids interacting with the inducing peptide. The position of the *AluI* and *RsaI* sites in *agrD* seems to be sufficient to assign a strain to a particular *agr* group, whereas restriction sites characteristic of each group can also be found in other genes of this region (Fig. 3). This adds to previous reports showing that genes of the *agr* locus are submitted to a coevolutionary pressure, allowing the binding of a modified AIP to the receptor (15, 22). Our classification indicates that type R VII-A12 and type R IX-A11 are particular with respect to the coevolution of the propeptide and its receptor. Indeed, the receptor-encoding genes of type R VII-A12 and R IX-A11 are highly similar to those of the *agr* group 1 strains, whereas their propeptide-encoding genes are highly similar to those of groups 3 and 2, respectively. The fact that the propeptide and its receptor belong to different interference groups suggests that strains of type R VII-A12 and type R IX-A11 are impaired in the activation of RNA III by the *agr* system. We postulate that these two types are in a process of evolution from group 1 to groups 2 and 3, respectively. We also identified a strain (type R VII-A3) which has a deletion of the complete *agrD* gene and which should thus also be impaired in the activation of RNA III by the *agr* system. Strains of types R VII-A12, R IX-A11, and R VII-A3 are nevertheless virulent, because they were all isolated from the milk of cows with mastitis. In connection with this, Wesson et al. showed that a strain mutated in *agr* was internalized by cultured bovine mammary epithelial cells at a level greater than the wild-type strain but contrary to the wild type failed to induce apoptosis (31). Others have also isolated virulent *S. aureus* strains with an inactivated *agr* system (30). These strains show increased adherence and biofilm formation, and these properties were considered important for the development of chronic infection (24).

We were also interested to know if the RAP-TRAP system is polymorphic and ubiquitously associated with *S. aureus* strains. We identified the *trap* gene in all strains analyzed, and we proved that at least four different alleles exist. We also tried to learn if different alleles of the RAP-encoding gene exist. As the nucleotide sequence of RAP is unknown, we made BlastN searches with the available RAP NH<sub>2</sub>-terminal sequence (IKKYKPITN). Curiously, homologies were only found with the well-conserved L2 ribosomal protein of *S. aureus* (results not shown). The identification of the TRAP activator thus needs further clarification, as it is difficult to understand how a conserved ribosomal protein is able to activate the two-component system. RNA III-inhibiting peptide (RIP), a peptide of sequence YSPXTNF, isolated from culture supernatants of a coagulase-negative *Staphylococcus* species that is believed to be *S. xyloso*, was found to compete with RAP on inducing TRAP phosphorylation. This leads to inhibition of RNA III synthesis and to diminution of the virulence phenotype (2, 3, 9). These results need now to be extended to strains belonging to each of the four *trap* types identified in this work. With the exception of the Newbould 305 and the NCTC 8325 strains that we classified as *trap* type 1, nothing is known about the allelic variation of *trap* in the strains previously tested for inhibition by RIP. As we have shown that most of the *S. aureus* strains (71.8%) are *trap* type 1, it can be speculated that most, if not all, of the strains tested for inhibition by RIP are also *trap* type

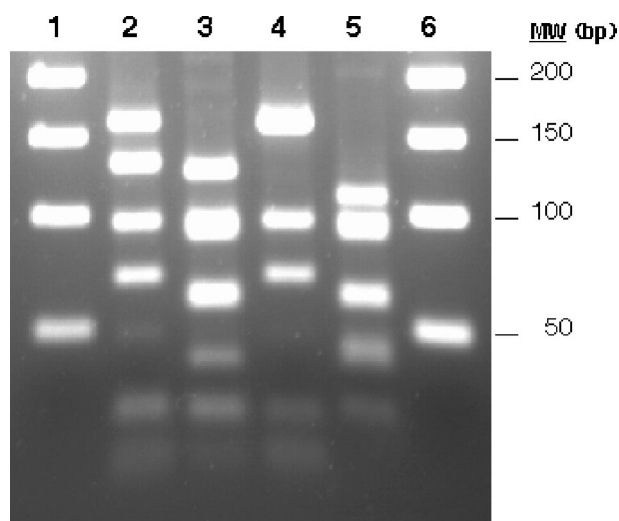


FIG. 5. Restriction polymorphism in the *trap* gene. The *trap* gene was amplified by PCR, digested with *MseI*, and electrophoresed on a 3% agarose gel. Examples of the different restriction types obtained are shown: type 1 (lane 2), type 2 (lane 3), type 3 (lane 4), and type 4 (lane 5). Molecular weight markers (50-bp DNA ladder; Promega) are shown in lanes 1 and 6.



1. It is thus still possible that RIP is not able to inhibit RNA synthesis in the other three *trap* types. Furthermore, it is also unknown if RAP purified from strains belonging to each of the four *trap* types are, as AIPs isolated from each of the four *agr* types, able to activate RNA III synthesis in strains of the same type and to inhibit this synthesis in strains of different types.

Most of the strains (56.3%) isolated from cows with mastitis belong to the *agr* R III-A1, *trap* 1 type. Our data indicate that strains belonging to this type have been able to infect cows from at least the end of the 1950s to date. The *agr* R III-A1, *trap* 1 type is also the type of the Newbould 305 strain (ATCC 29470), a strain isolated in the United States and widely used for experimental mastitis. This indicates that this type is not linked to a particular geographical location (under the circumstances of this work, France). The presence at a high prevalence of type *agr* R III-A1, *trap* 1 in the population of strains isolated from cows with mastitis suggests that this type has unique characteristics which, in contrast to the other rare types, endow it with superior ability to infect the bovine mammary gland. The *agr* R III-A1, *trap* 1 type is thus probably an *S. aureus* lineage that expands in the bovine population due to its possession of a unique combination of virulence genes. As no strain isolated from humans, whose *agr* sequence is available from GenBank, was found to belong to the R III-A1 type, it is tempting to think that on the contrary this *S. aureus* type is rarely isolated in the human population and that other *agr* and *trap* types are predominantly associated with human disease. This hypothesis will now be tested by analyzing the polymorphism of *agr* and *trap* in a population of *S. aureus* strains isolated from humans. Previous works using other methods also indicated that *S. aureus* isolated from humans and from cows has a predominantly clonal structure and that, whereas numerous types could be identified, only few of them are predominantly associated with a particular host and disease (1, 5–8, 13, 16, 33). The identification and characterization of a disease-dominant lineage(s) are very important for the development of vaccines and diagnostic tests. It could be expected that such works will lead in the future to the discovery of genetic determinants responsible for the tropism of different *S. aureus* lineages for specific hosts and tissues and to the development of new prophylactic and diagnostic tools.

### ADDENDUM

The sequence of the whole genome of MW2, a strain of community-acquired methicillin-resistant *S. aureus*, became available after this paper was submitted (GenBank accession no. AP004822 to AP004832). This strain falls into the classifications *agr* group 3 (type R VI-A8) and *trap* type 3.

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